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Plant Anaerobic Stress
II. Strategy of Avoidance of Anaerobiosis and Other Aspects of Plant Life under Hypoxia and Anoxia

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ABSTRACT

This review is a logical follow-up of previous publications (Vartapetian and Crawford 2007; Sachs and Vartapetian 2007) where an attempt was made to summarize the results of earlier periods of investigations of plant anaerobic stress and the activity of members of the International Society for Plant Anaerobiosis (ISPA) that ultimately brought about the establishment and international recognition of a new scientific discipline in the field of plant ecological physiology, biochemistry and molecular biology devoted to plant life under hypoxia and anoxia. Special attention was also paid to the strategy of metabolic adaptation of plants to hypoxia and anoxia, realized at the molecular level, including both the molecular biological and molecular genetic aspects of the problem. Continuing the discussion of strategies of plant adaptation to anaerobic environments in this review we pay particular consideration to the strategy of adaptation accomplished at the whole plant level by the formation of a continuous network of gas-filled spaces (aerenchyma), which development, provoked by specific signaling systems and programmed cell death, provides facilitated long-distance oxygen transport from aerated plant parts to organs (roots, rhizomes) under anaerobic conditions, that is a strategy of avoidance of anaerobiosis, or the phenomenon of "apparent" tolerance. Additionally, the following important aspects of plant hypoxic and anoxic stress are also considered here: post-anaerobic plant injury by reactive oxygen species and protection against oxidative injury by plant antioxidants; the Davies-Roberts pH-stat theory; alternative electron acceptors; demonstration of the adaptation syndrome in plants under anaerobic stress; and genetic and cellular engineering in generating plants tolerant to anaerobic stress.

Keywords: adaptation syndrome, aerenchyma formation, alternative electron acceptors, antioxidants, genetic and cellular engineering, oxygen translocation, reactive oxygen species
Abbreviations: **AA**, ascorbic acid; **ACC**, 1-aminocyclopropane-1-carboxylic acid; **CAT**, catalase; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **NMR**, nuclear magnetic resonance; **PCD**, programmed cell death; **PHGPH**, phospholipids hydroperoxide glutathione peroxidase; **ROS**, reactive oxygen species; **RNS**, reactive nitrogen species; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid reactive substances

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Invited Review

INTRODUCTION

Higher plants, which are aerobic organisms, frequently inhabit environments that are under conditions of temporal or permanent anaerobic stress (hypoxia and anoxia). Most often anaerobiosis takes place in flooded soils, as a result of low oxygen solubility and diffusion rate in water. Anaerobic stress substantially suppresses cell aerobic metabolism, and often results in severe damage ultimately leading to death of agricultural and wild plants (Hook and Crawford 1978; Kozlovsky 1984; Crawford 1987; Jackson *et al*. 1991; Jackson and Black 1993; Crawford and Braendle 1996).

However, through evolution and selection many plant species have become adapted to inhabiting waterlogged and even submerged soils when plants are partly or completely under water. According to contemporary views there are two general strategies of plant adaptation to anaerobic environments (Vartapetian 1978), namely metabolic adaptation, which is realized at the molecular level and is illustrated by a radical redirection of protein, carbohydrate and energy metabolism (Sachs and Vartapetian 2007) and anatomical-morphological adaptation at the whole plant level through its capacity to avoid anaerobiosis by translocating oxygen from aerated parts into submerged organs (roots, rhizomes; Armstrong 1978; Vartapetian *et al.* 1978a; Armstrong 1979; Vartapetian and Jackson 1997; Jackson and Armstrong 1999). The strategy of plant metabolic adaptation to hypoxia and anoxia was considered in our previous publication (Sachs and Vartapetian 2007). In the present review, emphasis is put on the general strategy of plant adaptation to anaerobic stress which is realized at the whole plant level by long-distance oxygen transport, a strategy of anaerobiosis avoidance. In addition, several other important aspects of plant life under hypoxia and anoxia are considered: post-anaerobic plant injury by reactive oxygen radicals; acidification of cell cytoplasm under anaerobic stress and its regulation; alternative electron acceptors under anoxia (nitrate and anaerobically synthesized lipids); visualization and demonstration of the adaptation syndrome in plants under anaerobic stress and possible molecular mechanisms that may be responsible for it; and genetic and cellular engineering approaches in generating plant cells and regenerated plants tolerant to low oxygen stress.

AVOIDANCE OF ANAEROBIOSIS

Higher plants avoid anaerobiosis in several ways; for instance by producing surface adventitious roots (Jackson and Armstrong 1999) or rapidly growing under water to make their way to the surface aerobic environment as shown for submerged wild plants such as *Potamogeton pectinatus*, *P. distinctus* and *Rumex palustris* (Summers *et al.* 2000; Sato *et al*. 2002; Voesenek *et al.* 2003) and deepwater rice (Kende *et al.* 1998; Almeida *et al.* 2003; Vriezen *et al*. 2003). Nevertheless, as noted by Jackson and Armstrong (1999) and Armstrong *et al.* (1994), the most widespread and efficient way for a plant to avoid anoxia is the development of a continuous gas-filled hollow network, aerenchyma, in the cortical tissue of roots, stems, and leaves, which facilitates oxygen transport by diffusion and mass flow (conversion) from aerated above-ground organs to those located in the anaerobic environment (down to the root tips).

Besides internal oxygen translocation facilitated by aerenchyma, partially submerged plants, for instance deepwater rice, avoid root anaerobiosis due external aeration (Raskin and Kende 1983, 1985; Becket *et al*. 1988). In experiments with deep-water rice Raskin and Kende (1983, 1985) presented the evidence for the existence of air layers between hydrophobic surface of submerged leaf and surrounding water. These air layers provide an aeration path which, according to these authors, is vital for partially submerged plants.

Therefore, we first consider the mechanism of aerenchyma formation and then compare oxygen transport in plants with developed aerenchyma and those without them.

FORMATION OF AERENCHYMA

Roots of plants growing in flooded soils are exposed to an environment devoid of oxygen and in which reduction processes prevail, leading to the accumulation of toxic inorganic and organic compounds in the soil and to the suppression of nitrification and nitrogen fixation (Ponnamperuma 1984; Gambrell *et al.* 1991; Blom 1999; Kirk and Kronzucker 2005). The development of aerenchyma in hydrophytes inhabiting flooded soils and mesophytes growing in dry soils as well as the role of aerenchyma in oxygen transport from aerated plant parts to organs under an anaerobic environment (roots, rhizomes) have been considered in detail in several reviews (Armstrong 1978, 1979; Drew 1992; Armstrong *et al.* 1994; Jackson and Armstrong 1999; Colmer 2003; Evans 2004).

The formation of continuous gas-filled spaces in above- and underground organs facilitate oxygen transport by diffusion and mass flow from aerated shoots to roots and rhizomes. In addition, aerenchyma supplies oxygen to the rhizosphere through diffusion from the roots towards the outside environment. This flow of oxygen is involved in the detoxification of reduced iron, manganese, and hydrogen sulfide, which accumulate in anaerobic soil (Ponnamperuma 1984; Gambrell *et al.* 1991). Oxygen secreted from the roots is also involved in nitrification and nitrogen fixation (Blom 1999; Kirk and Kronzucker 2005). The occurrence of aerenchyma favors the removal, with an ascending flow, of certain volatile compounds (ethylene, CO_2, and CH_4), which also accumulate in flooded soils. Methane, produced in anaerobic rice fields is one of the major compounds responsible for global climate warming on our planet (Neue *et al.* 1990). About 25-60 million tons of methane are emitted from rice fields into the atmosphere each year (Neue *et al*. 1990).

Aerenchyma develops most often in plants inhabiting flooded soils (Armstrong 1978; Armstrong *et al*. 1994). Under flooding-induced oxygen deficiency in the rhizosphere, when primary roots perish, aerenchyma is also formed in adventitious roots of many plants cultivated on dry soils (maize, wheat, sunflower, and clover) (Kawase 1981; Smirnoff and Crawford 1983; Jackson and Drew 1984; Jackson 1985; Campell and Drew 1983; Watkin *et al.* 1998; Aschi-Smith *et al.* 2003).

Aerenchyma is formed in plants constitutively by schizogeny or can be induced by low oxygen content by lysigeny. One or another mechanism of aerenchyma formation prevails in various plant species (Kawase 1981; Smirnoff and Crawford 1983; Jackson and Drew 1984; Jackson 1985; Armstrong *et al.* 1994; Justin and Armstrong 1991; Watkin *et al.* 1998; Jackson and Armstrong 1999; Aschi-Smith *et al.* 2003). In the case of schizogeny, gas-filled spaces are formed by controlled cell division and expansion. This is more characteristic of plants inhabiting excessively wet and flooded soils and under such circumstances the process is predominantly a constitutive event. In fact, the mechanism(s) underlying the development of schizogenous aerenchyma has yet to be fully addressed. Another better understood mechanism of aerenchyma formation is through se-

lective degradation of some cells in the cortex and is termed, lysigeny, i.e., programmed cell death (PCD) and it is mainly induced by low oxygen concentrations in the soil during such events as excessive rain, irrigation or flooding.

Signal factors in the formation of aerenchyma

During soil flooding, when oxygen content in both the roots and the rhizosphere drops, some biochemical processes precede cell death: one of the best characterized being the accumulation of ethylene in the both the roots and the rhizosphere (Drew *et al*. 1979; Kawase 1981; Jackson and Drew 1984; Jackson 1985; Watkin *et al.* 1998). This rise in ethylene results in the expression of the genes responsible for cell deg-radation and death. Ethylene, which increases substantially during oxygen deficiency due to soil flooding, is a signal molecule triggering a chain or cascade of events leading to aerenchyma formation (Brailsford *et al.* 1993; He *et al.* 1994, 1996). This has been shown in experiments in which aerenchyma formation could be arrested by the inhibition of ethylene synthesis or function and resumed by a treatment with exogenous ethylene (He *et al.* 1996). It has been shown that a decrease in the oxygen content in the rhizosphere primarily reflects upon ethylene content in the root stele, where anaerobic conditions start earlier than in the root cortex (Armstrong and Beckett 1987; Darvent *et al.* 2003; Garthwaite *et al.* 2004). Under conditions of hypoxia or anoxia in the root stele, the enzyme catalyzing the synthesis of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), is activated (He *et al.* 1994). Low oxygen levels are required for ACC conversion into ethylene. In the presence of a low concentration of oxygen (3.0-12.5%) in the root cortex, ACC oxidase is activated and ACC is converted into ethylene.

Induction of the above-mentioned biochemical processes under hypoxia and anoxia indicates that the cells sense a low oxygen level and activate a signaling cascade inducing genes encoding anaerobic proteins.

Earlier speculation suggested that nonsymbiotic haemoglobin (Hb) could help sense oxygen deficiency (Appleby *et al.* 1988). However, this supposition now seems rather unlikely because Hb binds oxygen tightly (dissociation constant 0.0272 s^{-1}, Duff *et al.* 1997), although it has been shown that the synthesis of nonsymbiotic Hb is strongly enhanced under hypoxia and the protein accumulates in the cells (Duff *et al.* 1997). The authors of these studies believe that metabolic pathways including the interaction between Hb and nitric oxide (NO) under hypoxia are an alternative route to mitochondrial electron transport during plant respiration, i.e., under such conditions, Hb functions like an dioxygenase of NO, which is formed under anaerobic conditions because of nitrate reduction (Igamberdiev *et al.* 2005). The authors hypothesized that stress-induced Hbs, functioning as dioxygenases detoxify NO and oxidizing NADH under oxygen deficiency, thus maintain the ATP level by an as yet unknown mechanism. Alfalfa plants over-expressing the Hb gene were more tolerant to flooding than either the wild-type plants or the plants with suppressed Hb expression (Dordas *et al.* 2003; Baron *et al.* 2004; Hill 2004).

NO• as a signaling molecule in plant tissues

The chemical properties of nitric oxide make this gas a good candidate as a signaling molecule. NO can freely penetrate the lipid bilayer, and, hence be transported within the cell. NO can be quickly produced on demand via inducible enzymatic and non-enzymatic routes. Due to its free radical nature (one unpaired electron) NO has a short half-life (in the order of a few seconds), and can be removed easily when no longer needed (reviewed by Lamattina *et al.* 2003 and Neill *et al.* 2003). Nitric oxide is represented by three species with different chemical reactivity and physical properties: radical NO•, nitrosonium cation (NO^+) and nitroxyl anion (NO^-). Nitric oxide can have direct or indirect biological effects; the direct effects take place at low NO concentrations (<1 μM) (Wink and Mitchell 1998), while the indirect effects through reactive nitrogen species (RNS) take place at higher local concentrations (>1 μM). The direct NO effects include the reduction of free metal ions or the oxidation of metals in protein complexes such as Hb, and Fe-nitrosyl formation thus resulting in the activation of guanylate cyclase and hemoxygenase and the inhibition of P450, cytochrome c oxidase and catalase, as well as the stimulation of T_fR protein and the down-regulation of ferritin (Wink and Mitchell 1998).

A number of investigations have been carried out on the involvement of NO during plant development (reviewed by Beligni and Lamattina 2001; Wendehenne *et al.* 2001, 2003; Gechev *et al.* 2006). NO has also been found to slow down plant senescence in pea leaves, in cut flowers and in ripening fruits (Leshem 2000) pointing towards NO and programmed cell death regulation. Furthermore, cytokinins have been shown to induce synthesis in tobacco, parsley and *Arabidopsis* cell cultures. Since a nitric oxide synthase (NOS)-inhibitor has been shown to hinder cytokinin-induced betalaine accumulation in *Amaranthus*, it has been suggested that NO takes part in the cytokinin signaling route in plant tissues (Tun *et al.* 2001, and references therein). Hence, NO may also mediate cytokinin-induced programmed cell death (Carimini *et al.* 2002). It has also been shown that NO induces programmed cell death needed for aerenchyma development via hydrogen peroxide (Borutaite and Brown 2003).

The large number of physiological and developmental effects of NO point towards regulation of gene expression (reviewed by Neill *et al.* 2003). This has indeed been observed in some occasions, e.g. in TMV-resistant tobacco NOS activity increases after infection (Klessig *et al.* 2000). In a microarray study on *Arabidopsis* suspension cultures it was shown that a number of genes are induced by NO and a common induction mechanism was suggested for some of the genes, although no data on a common regulatory element in the promoter areas of these genes exist as yet (Huang *et al.* 2002). More recently, it has been shown that NO and ROS induce changes in the transcription of many genes and work in a complementary manner. For example, phenylalanine ammonia lyase (PAL) and chalcone synthase are induced by NO without the involvement of ROS, while glutathione-*S*-transferase has been shown to be induced by H_2O_2 (Grün *et al*. 2006). Also, the combined effect of NO and H_2O_2 has been tested in a series of experiments on catalase-deficient tobacco mutants treated with NO and exposed to high light (Zago *et al.* 2006). The latter experiments proved that NO and H_2O_2 work together in the induction of programmed cell death.

Enzymatic sources of reactive nitrogen species (RNS)

In recent years, reactive nitrogen species and especially nitric oxide (NO) have become the focus of research in plant signaling. The best known route for NO production in plant tissues is through nitrate reductase. In the presence of nitrite and NADH and, under physiological pH levels nitrate reductases are capable of NO• and RNS production *in vivo* and *in vitro* in the absence of O_2 (Yamasaki and Sakihama 2000; Rockel *et al.* 2002). Activation of nitrate reductase under hypoxic conditions in barley roots, and accumulation of NO during hypoxic treatment in maize cells have been shown, and a role for NO as a signal for aerenchyma formation has been hypothesized (Dordas *et al.* 2003). The regulation of NO level under oxygen deprivation can be achieved in plants via interaction with stress-induced non-symbiotic Hb through several routes as described by Dordas *et al.* (2003).

In mammalian cells three types of nitric oxide synthases (NOS, EC 1.14.13.39) have been described: a constitutively expressed neuronal (nNOS), an endothelial (eNOS), both under the control of Ca^{2+}-calmodulin, and an inducible (immunological) iNOS. The isoforms are the products of dif-

ferent genes with 50-60% homology and share common co-factors and chemistry for NO production (Wendehenne *et al.* 2003). The functional NOS catalyzes oxygen dependent conversion of L-arginine to citrulline and NO (Alderton *et al.* 2001) according to the following reaction:

L-arginine + NADPH + O_2 → citrulline + NO• + $NADP^+$.

However, no plant homologue of mammalian NOS has been found in the *Arabidopsis thaliana* genome. At present, two pathways have been identified in plants for the production of NO, i.e. a nitrite-dependent route described above and an arginine-dependent route (Crawford 2006), while the actual functioning of the recently found *AtNOS1* gene has been questioned (Guo *et al.* 2003; Crawford *et al.* 2006; Guo 2006; Zemojtel *et al.* 2006). The product of this gene is known to be needed for NO synthesis *in vivo* and its biochemical properties are similar to mammalian constitutive cNOS, however, it bears no sequence similarity to known animal NOS. Another novel pathogen-induced enzyme with NOS activity has been identified in plants, and it appears to be a variant of the P protein of the glycine decarboxylase complex (GDC) (reviewed by Douce *et al.* 2001). This protein again shares little homology with mammalian NOS (Chandok *et al.* 2003). The *Arabidopsis thaliana* P protein of the GDC complex is 89% identical to this variant P protein with NOS-like activity from tobacco. However, the poor homology to mammalian NOS may suggest an alternative pathway for NO production (Chandok *et al.* 2003).

Xanthine oxidoreductase (XOR), a redox enzyme with a Mo cofactor, is another inducible source of NO in the context of stress responses in mammals. At low oxygen tensions the NO-generating activity of this enzyme is increased. Interestingly, under normoxic conditions xanthine oxidoreductase is capable of both NO• and O_2^-• formation with consequent production of $ONOO^-$ (Godber *et al.* 2000).

However, whether XOR produces NO in plants is not yet established.

Non-enzymatic sources of NO

The formation of NO via non-enzymatic reduction of exogenous nitrite has been shown in the apoplast of barley (*Hordeum vulgare*) aleurone layers. The process requires acidic pH and its rate is enhanced by phenolic compounds (Bethke *et al.* 2004). Non-enzymatic NO production can be a factor under pathological conditions, i.e. hypoxia, which is characterized by cytoplasmic acidosis and accumulation of reducing equivalents in both animal and plant systems (Dordas *et al.* 2003).

Calcium as a signaling factor

In experiments with maize plants and cell cultures, Sachs and coworkers (Subbaiah *et al.* 1994; 1998; 2000; Subbaiah and Sachs 2003) demonstrated an immediate involvement of calcium ions as a signal factor during the very early stages of aerenchyma formation. Under oxygen deficiency, calcium is released from the apoplast and from mitochondria into the cytoplasm, provoking the subsequent activation of kinases and phosphatases, resulting in the activation of genes responsible for the synthesis of ethylene and subsequent reactions leading to the cell death. Mitochondria also take part in the induction of programmed cell death through the release of cytochrome c as a proapoptotic signal (Virolainen *et al.* 2002). It has been shown in particular that under oxygen deficiency after the calcium signal, ethylene synthesis is enhanced, and this leads to cell walls being degraded by cellulase, pectinase, and xylanase (Kawase 1979; Grineva and Bragina 1993; Grineva *et al.* 2000; Gunawardena *et al.* 2001a, 2001b; Bragina *et al.* 2003), and also probably xyloglucan endotransglycosylase (XET), which destroys cell wall xyloglucans (Saab and Sachs 1996).

Plant cells perish by apoptosis (programmed cell death or PCD), under mechanisms that appear to be similar in animals and plants. The first PCD signal being increased cytoplasmic calcium, which quickly leads to the release of proapoptotic signals if other conditions are favorable for PCD (Drew 1997; Gunawardena *et al.* 2001a, 2001b; Chichkova *et al.* 2004; reviewed by Drury and Gallois 2006).

Aerenchyma formation and PCD

Under hypoxia (at partial submergence, i.e. roots only under water), inner cortical cell layers of the primary or nodal roots are selectively killed leading to aerenchyma formation. This selective cell death not only reduces the demand for O_2 but more importantly, enhances root porosity and facilitates oxygen diffusion from the exposed plant parts toward submerged ones. Aerenchyma formation requires the presence of some oxygen (hypoxia) and occurs 3-4 cm behind the tip (He *et al.* 1992). This enhances the survival of the root (Gibbs *et al*. 1995), and the prolonged survival of the seedlings. The nature and regulation of cell death during aerenchyma formation has been analyzed (He *et al.* 1992; Drew *et al*. 2000; Gunawardena *et al*. 2001a, 2001b; Evans 2004). These various studies indicate that aerenchyma formation is under genetic control (reviewed in Drew *et al*. 2000). Cytohistological data, however, indicate that the hypoxically-induced PCD does not entirely follow the canonical apoptotic pathway reported for animal cells, but partly resembles cytoplasmic or necrotic death (Gunawardena *et al*. 2001b).

Root-tip death

Under complete submergence (or being subjected to immediate strict anoxia; i.e., 'anoxia shock'), maize seedlings exhibit another cell death process that also appears to have an adaptive significance. Although prolonged anoxia ultimately kills the entire seedling, different tissues of an individual plant differ in their tolerance (Vartapetian *et al.* 1978a, 1987; Johnson *et al*. 1989; Ellis *et al*. 1999). Root tips in maize, as in other plants, are very sensitive to anoxia and die within a few hours (Vartapetian *et al.* 1970, 1977, 1978a; Roberts *et al*. 1984b; Johnson *et al*. 1989; Folzer *et al*. 2006; Gladish *et al*. 2006). Root tips are composed of tightly packed tissues with few, if any, intercellular spaces and therefore suffer from restricted gaseous diffusion. Consequently, in flooded seedlings, root tip death may be a natural consequence of oxygen starvation and the attendant repression of substrate transport. Considerable attention has been given to strategies/mechanisms that prolong the anoxia tolerance of the primary root tip in young maize seedlings, as the tip of the primary root has been considered to be very important for seedling establishment (Drew *et al*. 1994). On the other hand, it was proposed that under severe anoxia, when energy generation is extremely limiting, the loss of metabolically actively intensive tissues such as the root-tip might prolong the survival of the shoot and the root axis. The facilitated survival of these two organs (shoot and root) during submergence may increase the chances of seedling recovery after reoxygenation. This was examined and results indicate that the root tip indeed acts as a dispensable and non-functional sink in anoxic seedlings (Subbaiah *et al*. 2000; Subbaiah and Sachs 2001). Excision of the root tip (de-tipping) before anoxia led to a superior recovery of seedlings from stress injury. De-tipped seedlings showed lesser root axis damage and an increased production of lateral roots compared to intact seedlings (Subbaiah *et al*. 2000).

An anaerobically induced polypeptide, sucrose synthase (SUS-SH1), was shown to be post-translationally regulated by phosphorylation, and this regulation is among the early responses that culminate in the death of primary root tip in anoxic maize seedlings (Subbaiah and Sachs 2001). Sucrose synthase (SuSy; SUS) is a unique enzyme with an ability to mobilize sucrose into diverse pathways that are critical in structural (e.g., cellulose or callose biosynthesis), storage (starch synthesis) and metabolic (e.g., glycolysis) functions of plant cells (e.g., Ruan *et al*. 1997). It is encoded by three genes in maize, *sh1* (encoding SUS-SH1; Chourey and Nelson 1976), *sus1* (encoding SUS1; Chourey 1981; Chourey *et al*. 1998) and *sus2* (encoding SUS2; Carlson *et al.* 2002;

Chourey 2006). The *sh1* gene is expressed mostly in the developing endosperm, whereas *sus1* is expressed in many plant parts including the aleurone and basal part of the developing endosperm. The *sh1* gene is induced by anoxia both at transcriptional and translational levels (ANP87; Springer *et al*. 1986). The *sus1* gene is only mildly induced by anoxia. Although the double mutants in of *sh1* and *sus1* have been shown to be less tolerant to anoxia (Ricard *et al*. 1998).

Under anoxia, the phosphorylation state of SuSy encoded by *sh1* is correlated with membrane localization in maize primary root tips. This localization is correlated with callose accumulation and is associated with death of the root tip. Maize *sh1* mutants showed sustained SuSy phosphorylation and did not exhibit the relocation to the root tip membranes and had less callose accumulation and greater tolerance to prolonged anoxia than their non-mutant siblings (Subbaiah and Sachs 2001). In addition to its functions in directing sucrose toward glycolysis and fermentation and in root tip death, SuSy apparently has other functions in responses to flooding stress that stem from its mitochondrial localization (Subbaiah *et al.* 2006).

Another enzyme that appears to be involved in the root tip death phenomenon is an anoxia-induced protease (AIP). This protease is the predominant proteolytic activity in the root tip during anoxia. Furthermore, the superior anoxia tolerance of de-tipped seedlings is associated with a decreased AIP activity. Thus, the appearance of AIP activity in the root tip during anoxia is spatially and temporally associated with the root tissue death (Subbaiah *et al*. 2000).

These studies indicate that the root tip elimination early during anoxia may provide an adaptive advantage and that maize may be evolving with the *sh1*-encoded SuSy and the anoxia-induced protease systems, a mechanism to induce cell death in the root tip as a means of tolerance to flooding.

Root tip death under anoxia: programmed cell death or necrosis?

Cell death is a basic biological process important in the regulated development of multicellular organisms and in their responses to stress. Animal cells show two fundamentally different modes of cell death, namely apoptosis (or PCD) and necrosis. The most relevant distinction between the two types of death is the early preservation of membrane integrity in apoptosis, whereas a rapid release of intracellular constituents occurs in the case of necrosis. Therefore, necrosis can presumably be dangerous, while the apoptotic process is an adaptive mechanism to dispose of cells without compromising the integrity of the organism. Nevertheless, increasing evidence points to the fact that apoptosis and necrosis represent just extremes of a wide range of possible morphological and biochemical cell death processes. Root tip death is preceded by SH1 relocation, DNA nicking, and induction of AIP as well as callose, indicating that the process, to some extent, is autonomous (and a programmed event). On the other hand, the death of root tip cells is accompanied by the acidification of the cytosol (Roberts *et al*. 1984a; Kulichikhin *et al*. 2007) as well as the external medium and an extracellular release of diffusible cytotoxins (Subbaiah *et al*. 1999). Therefore, root tip death in nature may be a less cell-autonomous but more of a necrotic process (Van Breusegem and Dat. 2006). De-tipping experiments (Subbaiah *et al*. 2000) suggest that an acceleration of the process as well as making it more cell-autonomous (i.e., pushing the process more towards PCD) would provide a definite advantage during post-anoxic recovery of maize seedlings.

The essence of stress adaptation is redirecting scarce resources to the maintenance of essential sinks as well as activation of adaptive pathways, while disinvesting in non-essential sinks and pathways. Being endowed with multiple growing points, plants have a unique ability to eliminate superfluous tissues/organs under stress and regenerate them if favorable conditions appear again. O_2-deprived maize roots exhibit two such regulated cell or tissue-death pathways. These two pathways are clearly distinct in their regulation as well as the location of their occurrence in the root.

Therefore, a reprogramming of root tip death to have it occur early, during anoxia, may provide a definite adaptive advantage to maize seedlings exposed to anoxic stress. In *Arabidopsis*, the whole root system is dispensable for hypoxic tolerance of the seedlings; in fact, de-rooted seedlings did better under O_2 deprivation (Ellis *et al*. 1999). In maize, the primary root axis is necessary (in quickly generating a functional root system), if not essential, for the post-anoxic recovery of seedlings. However, the survival of the shoot meristem is critical for the post-anoxic re-growth and autotrophic life of the seedling.

OXYGEN TRANSLOCATION

The results of earlier investigations on oxygen translocation from aerated above-ground plant tissues to anaerobically located roots have been considered by Armstrong (1978) and Vartapetian *et al.* (1978a) in the monograph edited by Hook and Crawford (1978) and more recently in the review of Sachs and Vartapetian (2007). Here we recall some basic and essential findings. In dry-land mesophyte plants, such as, for example, cotton (*Gossypium hirsutum*; 22-26 days; 27°C), oxygen transport comprises only a small portion (7%) of root requirements in oxygen under aerobic condition (Nuritdinov and Vartapetian 1981). The proportion of O_2 transport increases only at low temperature, attaining, in cotton for instance 27% at 10°C. In experiments with *Alnus glutinosa*, exploring the effects of pressurized ventilation, diffusion and photosynthesis on root aeration Armstrong and Armstrong (2005a) also came to conclusion that low temperature helped to improve root aeration. The above mentioned phenomenon observed in experiments with *Gossypium hirsutum* and *Alnus glutinosa* could be explained as a result of marked drop in oxygen requirement for root respiration at low temperature without a substantial decrease in its translocation from shoots to roots. In the case of mesophytes tested, oxygen did not diffuse markedly from the roots into the external solution, at least, in experiments reported by Vartapetian and coworkers (Vartapetian *et al.* 1978a).

Nevertheless, the results obtained permitted to conclude that even very low levels of oxygen transport plays a definite protective role in the anaerobically incubated root life of mesophyte plants. Electron-microscopic examination of detached root mitochondria showed that degradation of their ultrastructure occurred within 6 to 10 h of the start of anaerobic incubation. Whereas in the roots of control intact plants, mitochondrial ultrastructure was maintained much longer (two to three days) in anaerobic environments (Vartapetian *et al.* 1978a; Andreeva *et al.* 1979). It was demonstrated that not only oxygen but also assimilates coming from shoots could play a definite role in the tolerance of intact roots to anoxia. In fact, in special experiments with cotton, ^{14}C-sucrose transport from aerated organs to anaerobic roots was studied (Vartapetian *et al.* 1978a; Nuritdinov and Vartapetian 1980). Results showed that such transport occurred during a rather long-term anaerobic incubation of roots, although its rate was substantially reduced substantially with time. Therefore, early degradation of cell ultrastructure in detached roots could be to some degree induced by an exhaustion of substrates for glycolysis and alcoholic fermentation, i.e., substrate starvation. In fact, the imitation of assimilate transport into plant detached plant roots by feeding them glucose considerably improved their tolerance even to strict anoxia (Vartapetian *et al.* 1977, 1978a). Feeding even intact roots with exogenous glucose under conditions of anaerobiosis also favored ^{14}C-sucrose inflow from leaves (Nuritdinov and Vartapetian 1980). Finally, a much higher tolerance to anoxia of intact roots as compared with detached roots is probably explained by hypoxic acclimation of intact roots occurring due to limited oxygen transported from aerated organs.

The usage of polarographic techniques for measurements of molecular oxygen translocation and mathematical models showed that in plant-inhabiting flooded soils (for instance, rice) as oppose to mesophytes (pumpkin, cotton) that are cultivated on aerated dry soils, oxygen was easily transported from above-ground aerated organs to oxygenate both root cells and the rhizosphere (Armstrong 1970; Vartapetian *et al*. 1970, 1978a; Armstrong 1979). This is in good agreement with electron-microscopic studies (Vartapetian *et al.* 1970, 1978a) and also with the results of physiological and biochemical investigations of Webb and Armstrong (1983) and ap Rees's laboratory (Ap Rees and Wilson 1984; Ap Rees *et al*. 1987) on the hypersensitivity of rice and other hydrophyte roots to oxygen deficiency. In the above mentioned experiments, it was confirmed that the roots of tolerant plants (rice, *Glyceria maxima*) inhabiting flooded soils were really more sensitive to oxygen deficiency than the roots of plants sensitive to flooding (pea, pumpkin).

Finally, the results of electron-microscopic, biochemical, and physiological investigations (Vartapetian *et al.* 1970, 1978a; Webb and Armstrong 1983; Ap Rees and Wilson 1984; Ap Rees *et al.* 1987) were confirmed in experiments with hydrophytes constantly living on flooded soils (Vartapetian and Andreeva 1986). It was demonstrated that feeding with exogenous glucose to anaerobically incubated roots of hydrophytes *Carex leporina*, *Alisma plantago-aquatica*, and *Lycopus europaeus* did not improve their adaptive properties, as it was found for the roots of mesophyte pumpkin (Vartapetian *et al.* 1977, 1978a) grown on dry soils. Thus, the roots of these hydrophytes being sufficiently supplied with oxygen transported from above-ground organs did not develop in the course of evolution the protective defense molecular mechanisms of adaptation to oxygen deficiency. In view of these findings, it is interesting to consider the results obtained by Crawford (1978) who demonstrated that, as oppose to roots of plants living on dry soils, the anaerobically incubated roots of hydrophytes, exhibited neither ADH activation nor an acceleration of alcoholic fermentation.

Thus, the results of the above-mentioned studies (Vartapetian *et al*. 1970, 1978a; Webb and Armstrong 1983; Ap Rees and Wilson 1984; Ap Rees *et al.* 1987) led to the paradoxical conclusion that the roots of plants constantly inhabiting flooded anaerobic soils are less, or not at all, metabolically adapted to anoxic environments. As opposed to roots of hydrophytes, those of mesophytes, which are exposed to oxygen deficiency only occasionally, developed some adaptive mechanisms.

The situation with root aeration becomes much more complex when shoots are submerged as well, as occurs with rice seedlings in East and South-East Asia (Setter *et al.* 1997; Jackson and Ram 2003; Mohanty and Ong 2003) or with some submerged wild plants capable of active growth under water (Summer*s et al.* 2000; Sato *et al.* 2002; Voesenek *et al.* 2003; Voesenek and Peeters 2004). When plants are completely submerged, oxygen supply to shoots and especially to roots declines sharply. Photosynthetic oxygen formation within the plant is also suppressed in submerged plants because of a lower availability of atmospheric CO_2 and a reduced plant illumination, especially when the plants grow in deep or muddy waters (Setter *et al.* 1997). These limitations result in a decreased photosynthesis and thus a poor root photoassimilate supply as well. This is especially true during night hours (darkness), particularly in rice, where the roots suffer from oxygen deficiency and switch to alcoholic fermentation (Waters *et al.* 1989; Boamfa *et al.* 2003; Pedersen *et al.* 2004). Root growth ceases under such conditions. Nevertheless, green parts of submerged plants are capable of photosynthesis under water, which alleviates substantially such severe conditions, providing plants with both oxygen and assimilates at least during hours of daylight (Mohanty and Ong 2003; Mustroph *et al.* 2004; Mommer and Visser 2005*)*.

When some leaves emerge from the water and are in contact with the atmosphere, as occurs for instance with deep-water rice (Armstrong *et al.* 1994; Kende *et al.* 1998; Almeida *et al.* 2003; Vriezen *et al.* 2003) the situation is more favorable because the roots obtain oxygen from both the leaves via aerenchyma and over the leaf blade surface (Raskin and Kende 1983, 1985; Beckett *et al*. 1988). According to Raskin and Kende (1983, 1985) continuous air layers trapped between hydrophobic corrugated surface of the leaf blades of deep water rice and surrounding water constitute the major path of aeration. This results in an extremely rapid growth (20-30 cm per day) of deep-water rice for instance during monsoon periods. As a result of such high growth rates, a continuous contact of some deep-water rice leaves with the atmosphere is preserved despite several meters of water covering the plants. Rapid growth of some submerged wild plants, *Potamogeton* and *Rumex* species, for example, has also been described; these plants grow in water their leaves rise above the water surface due to intense spending of storage carbohydrates and a 3- to 6-fold increase in the rate of glycolysis (Pasteur effect) (Summer*s et al.* 2000; Sato *et al.* 2002; Voesenek *et al.* 2003; Voesenek and Peeters 2004).

In studies performed in the laboratory of Armstrong (Darvent *et al.* 2003), platinum oxygen microelectrodes were used to compare specific features of oxygen transport in plants with aerenchyma in roots and those devoid of them. Microelectrodes were inserted into the primary roots of maize at various locations along the root length and at various depths inside the root in order to evaluate a topology of oxygen distribution within the root in both the longitudinal and radial planes. The results of these investigations confirmed the notion that the root cortex is its most aerated part comprising the channels for oxygen transport, whereas the stele is the least aerated part (Thomson and Greenway 1991). When oxygen content in the root environment declines, the anaerobic conditions arise first in the stele, resulting in the activation of synthase of ACC, a precursor for ethylene, and the subsequent synthesis of ethylene with the involvement of the cortex located ACC oxidase. In roots devoid of aerenchyma (wheat, for example), a decrease in the oxygen level occurs along the root length, and at the depth of more than 10 cm, oxygen essentially could not be detected in the root tips, which led to their death because of oxygen shortage. Hence it is clear why the roots devoid of aerenchyma penetrate soil no deeper than 10 cm (Thomson *et al.* 1990). As was demonstrated in Armstrong's laboratory (Darvent *et al.* 2003), in maize roots containing aerenchyma, the pattern of longitudinal and radial oxygen distribution in the root is quite different. Under anaerobic conditions, such roots are much better provided with oxygen transported from above-ground organs. This was further demonstrated in biochemical studies when adenylate energy charge was compared in anaerobically incubated roots with and without aerenchyma (Drew *et al.* 1985).

Thus, subsequent studies summarized in several publications (Vartapetian 1993a, 1993b; Armstrong *et al*. 1994; Jackson *et al.* 1999; Darvent *et al*. 2003; Vartapetian *et al.* 2003) confirmed and substantially added to the concept proposed earlier based on the electron microscopic, polarographic and chemiluminescent examinations as well as mathematical modeling of oxygen translocation in tolerant and sensitive plants (Armstrong *et al.* 1970, 1978, 1979; Vartapetian *et al.* 1970; Vartapetian 1973; Vartapetian *et al.* 1974, 1978a). Indeed these studies emphasized that, as distinct from mesophytes growing on dry soils, the principal strategy of adaptation of hydrophytes inhabiting flooded anaerobic soils is avoidance of root an-aerobiosis through long-distant oxygen transport but not through metabolic adaptation.

Furthermore the facilitated long-distance oxygen transport to the root tip in plants inhabiting flooded soils is provided, on the one hand, by the formation of expanded gas-containing spaces and, on the other hand, by impermeability of the basal root part to oxygen diffusion toward the rhizosphere (Armstrong and Beckett 1987; Jackson and Arm-

strong 1999; Garthwaite 2004). In experiments with seedlings of several rice varieties (Colmer *et al.* 1998; Colmer 2003), it was shown that in aerobically grown roots, the basal root parts of almost all varieties secreted oxygen into the rhizosphere. When roots were grown in oxygen deficit environment, in stagnant water, oxygen diffusion from basal root parts ceased thus facilitating its delivery to the root tip. Studying of sulfide–induced barriers to rice root radial oxygen loss Armstrong and Armstrong (2005) demonstrated marked root cell wall suberization and thickening correlated with reduced permeability to oxygen.

Albrecht and Mustroff (2003) showed that, under conditions of hypoxia, an enhanced synthesis of cellulose and callose in wheat roots was determined by the activation of sucrose synthase. According to these authors, an activated synthesis of these compounds helped to strengthen the cell walls, which counteracted tissue injury at a low oxygen content. In experiments by Armstrong and Armstrong (2005b) with rice roots submerged in non-running water, sulfides sharply improved the root barrier properties. As a result, radial oxygen secretion from root into the rhizosphere was considerably reduced, water uptake by roots was suppressed, and the growth of lateral roots was retarded.

POST-ANAEROBIC DAMAGE AND ADAPTATION

Plants suffer not only from anaerobic stress itself but also in the period when they are returned to normal conditions of oxygen supply after a short-term or long-term anaerobiosis (oxidative stress). This is due to two different issues: first, electrons accumulated in the cell respiratory chain under oxygen deficiency are transferred to molecular oxygen with the generation of reactive species (superoxide ion, hydrogen peroxide), which attack fatty acid unsaturated bonds in membrane lipids, denature proteins and nucleic acids, thus damaging plant cells substantially. Secondly, the antioxidative capacity of cell is weakened during oxygen deficiency, which increases the damaging affect of the reactive oxygen species, ROS (Blokhina *et al.* 2000). It has also been shown that many stress situations lead to increased production of superoxide, which is mitigated experimentally by overexpressing SOD (Yan *et al*. 1996, Lee *et al.* 2007a).

Thus, along with carbohydrate and energy shortage and cytoplasmic acidification during anaerobic stress, plants are subjected to a serious danger in the post-anoxic period. As in the case of energy shortage and cytoplasmic acidification, tolerant plants have developed defense mechanisms neutralizing adverse effects of free oxygen radicals in the post-anaerobic period. This topic has been discussed in detail in a review of Blokhina *et al*. (2003); therefore, we only briefly consider it below.

Sources of reactive oxygen species (ROS) in plant cells

As several review articles have been published recently both on the production of ROS and their scavenging by the many plant antioxidants as well as on the damage they may cause (Blokhina *et al.* 2003; Pitzschke *et al*. 2006), their production is but briefly described here, and the emphasis is placed on their signaling role in events during and after low oxygen stress. Generation of ROS is characteristic of all living tissues and cells and the delicate balance between their formation and quenching is strongly affected by low oxygen stress conditions. In the next paragraphs the focus is put on the new information emerging on the roles of ROS and reactive nitrogen species (RNS), molecules ideally suited to act as signaling molecules during oxygen stress conditions. To date, ROS and RNS are known to play key roles in signaling during both biotic and abiotic stresses as well as during developmental processes (e.g. systemic acquired resistance, ozone stress, temperature extremes, stomatal closure, senescence) and their action is strongly suggested in PCD taking place in aerenchyma formation (Bouranis *et al.* 2003; Van Breusegem and Dat 2006; Bouranis *et al.* 2007).

The initial step in ROS production requires initiation (one electron reduction), while subsequent reduction steps can proceed spontaneously in the presence of appropriate electron donors (Halliwell 2006). In plants the electron transport chains of chloroplasts and mitochondria are the main sources of electrons together with transition metal ions (Fe^{2+}, Cu^{2+}) and semiquinones. The highly reactive singlet oxygen ($^1O_2$) is produced in tissues under UV-exposure and during photoinhibition in chloroplasts, while hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) are both produced in a number of cellular reactions including the Mehler reaction in the chloroplasts, the iron catalyzed Fenton and Haber Weiss reactions, photorespiration and by various enzymes such as lipoxygenases, peroxidases, NADPH oxidase and xanthine oxidase. O_2^- is too reactive to pass membranes and is converted to H_2O_2 by compartment specific superoxide dismutase (SOD) isoforms.

The H_2O_2 molecule is relatively stable and less reactive than O_2^-, and is able to cross the lipid bilayer, a property which makes it a good candidate as a signaling species. It has been suggested also that H_2O_2 may pass the membrane through aquaporins, peroxide channels or other channels (Henzler and Steudle 2000; Ye and Steudle 2006). If so, the delivery of the H_2O_2 signal to a particular site can be indirectly regulated via aquaporin manipulation and, to some extent can solve the question of ROS signal specificity. It remains to be seen whether there are specific receptors for H_2O_2 in the plant cell.

A very reactive oxygen species, the hydroxyl radical OH•, is produced during the decomposition of ozone in the presence of protons in the apoplastic space and also in defence against pathogens (Bolwell *et al*. 2002), while the perhydroxyl radical O_2H• can be produced in a reaction of ozone with hydroxyl ions.

Antioxidant systems

In plant tissues the adverse effect of free radicals is controlled by the presence of low-molecular-weight endogenous antioxidants as well as antioxidant enzymes. The first do not only include ascorbic acid, glutathione, and tocopherols, but also many phenolic compounds which can act as antioxidants. In antioxidant turnover the corresponding enzyme systems reducing oxidized forms of antioxidants are of importance (for a review, see Noctor and Foyer 1998). The second include enzymes interacting with reactive oxygen species, SOD, peroxidase and catalase, and thus blocking ROS action. There are also a number of enzymes detoxifying lipid peroxidation products (glutathione *S*-transferases, phospholipid-hydroperoxide glutathione peroxidase and ascorbate peroxidase).

Low molecular weight antioxidants

Glutathione. Glutathione is a tripeptide (glutamylcysteinylglycine) and it is an abundant compound in plant tissues present in virtually all cell compartments: cytosol, ER, vacuole and mitochondria (Jimenez *et al*. 1998). GSH executes multiple functions and together with its oxidized form (GSSG) glutathione maintains the cellular redox balance. The latter property is of great biological importance, since it allows fine-tuning of the cellular redox environment under normal conditions and upon the onset of stress, and provides the basis for GSH stress signaling. Indeed, the role for GSH in redox regulation of gene expression has been described in many papers (e.g. Wingate *et al*. 1988; Alscher 1989). Due to redox properties of the GSH/GSSG pair and reduced SH-group of GSH, it can participate in the regulation of the cell cycle (Sanchez-Fernandez *et al.* 1997). The functioning of GSH as antioxidant under oxidative stress has received much attention during the last decade. It scavenges cytotoxic H_2O_2, and reacts non-enzymatically with other ROS: singlet oxygen, superoxide radical and hydroxyl radical (Larson 1988). The central role of GSH in the anti-

oxidative defense is due to its ability to regenerate another powerful water-soluble antioxidant, ascorbic acid, via ascorbate-glutathione cycle (Foyer and Halliwell 1976; Noctor and Foyer 1998).

Ascorbic acid (Vitamin C) is one of the most studied and powerful antioxidants (Noctor and Foyer 1998; Arrigoni and de Tullio 2000; Horemans *et al*. 2000; Smirnoff 2000). It has not only been detected in the majority of plant cell types and cellular organelles, but also in the apoplast. Under physiological conditions ascorbic acid (AA) exists mostly in its reduced form (90% of the ascorbate pool) in leaves and chloroplasts (Smirnoff 2000); and its intracellular concentration can build up to the millimolar range (e.g. 20 mM in the cytosol and 20-300 mM in the chloroplast stroma (Foyer and Lelandais 1996). The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes AA the main ROS-detoxifying compound in the aqueous phase. AA can directly scavenge superoxide, hydroxyl radicals and singlet oxygen and reduce H_2O_2 to water via ascorbate peroxidase reaction (Noctor and Foyer 1998). In chloroplasts AA acts as a cofactor of violaxantin de-epoxidase thus sustaining dissipation of excess excitation energy (Smirnoff 2000). AA regenerates tocopherol from tocopheroxyl radicals thus providing membrane protection (Thomas *et al*. 1992). In addition, AA carries out a number of non-antioxidant functions in the cell. It has been implicated in the regulation of the cell division, cell cycle progression from G1 to S phase (Liso *et al*. 1988; Smirnoff 1996) and cell elongation (de Tullio *et al*. 1999).

Tocopherol (Vitamin E). The importance of tocopherols and tocotrienols lies in the fact that they are essential components of biological membranes where they have both antioxidant and non-antioxidant functions (Kagan 1989). α-Tocopherol with its three methyl substitutes has the highest antioxidant activity of tocopherols (Kamal-Eldin and Appelqvist 1996). The other three tocopherol and tocotrienol isomers are (β-, γ-, δ-). Tocopherols and tocotrienols consist of a chroman head group and a phytyl side chain giving vitamin E compounds an amphipathic character (Kamal-Eldin and Appelqvist 1996). Though antioxidant activity of tocotrienols vs. tocopherols has been less studied, α-tocotrienol is proven to be a better antioxidant than α-tocopherol in the membrane environment (Packer *et al.* 2001). Tocopherols, synthesized only by plants and algae, are found in all plant parts (Janiszowska and Pennock 1976). Chloroplast membranes of higher plants contain α-tocopherol as the predominant tocopherol isomer, and are hence well protected against photooxidative damage (Fryer 1992).

The fact that makes Vitamin E especially important during the postanoxic phase in plant tissues is its chain-breaking antioxidant activity: It is able to repair oxidizing radicals directly, preventing the chain propagation step during lipid autoxidation (Serbinova and Packer 1994). It reacts with alkoxyl radicals (LO•), lipid peroxyl radicals (LOO•) and with alkyl radicals (L•), derived from PUFA oxidation (Buettner 1993; Kamal-Eldin and Appelqvist 1996). The reaction between vitamin E and lipid radicals occurs in the membrane-water interphase where vitamin E donates a hydrogen ion to the lipid radical with the consequent formation of tocopheroxyl radical (TOH•) formation (Buettner 1993). Regeneration of the tocopheroxyl radical back to its reduced form can be achieved by vitamin C (ascorbate), reduced glutathione (Fryer 1992) or coenzyme Q (Kagan *et al.* 2000). In addition, tocopherols may act as chemical scavengers of oxygen radicals, especially singlet oxygen, and as physical deactivators of singlet oxygen by charge transfer mechanism (Fryer 1992).

Phenolic compounds as antioxidants. Phenolics (flavonoids, tannins, hydroxycinnamate esters and lignin) are the largest group of secondary compounds in many plant tissues (Grace and Logan 2000). Polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), as well as their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans *et al*. 1997). Another mechanism underlying the antioxidative properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora *et al*. 2000). These changes could sterically hinder diffusion of free radicals and restrict peroxidative reactions. Moreover, it has been shown that phenolic compounds can be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahama and Oniki 1997).

Enzymes participating in quenching ROS

Superoxide dismutase (SOD)

Enhanced formation of ROS under stress conditions may induce both protective responses and cellular damage. The scavenging of $O_2^{\bullet -}$ is achieved through the upstream enzyme – SOD, which catalyses the dismutation of superoxide to H_2O_2. This reaction has a 10,000-fold faster rate than spontaneous dismutation (Bowler *et al*. 1992). The enzyme is present in all aerobic organisms and in all subcellular compartments susceptible of oxidative stress (Bowler *et al.* 1992). These enzymes, classified by their metal cofactor, can be found in living organisms; they are the structurally similar FeSOD (prokaryotic organisms, chloroplast stroma) and MnSOD (prokaryotic organisms and the mitochondrion of eukaryotes); and the structurally unrelated Cu/ZnSOD (cytosolic and chloroplast enzyme, Gram-negative bacteria). These isoenzymes differ in their sensitivity to H_2O_2 and KCN (Bannister *et al*. 1987). All three enzymes are nuclear encoded, and SOD genes have been shown to be sensitive to environmental stresses, presumably as a consequence of increased ROS formation. This has been shown in an experiment with corn (*Zea mays*), where a 7-day flooding treatment resulted in a significant increase in TBARS content, membrane permeability and the production of superoxide anion-radical and hydrogen peroxide in the leaves (Yan *et al*. 1996). In roots the activity of SOD was determined without a prolonged re-oxygenation period, immediately after termination of the anoxic treatment. Excessive accumulation of superoxide due to the reduced activity of SOD under flooding stress was also shown (Yan *et al*. 1996). On the whole, antioxidant defenses are induced in plants under mild oxidative stress conditions (Lee *et al.* 2007), while a severe stress, such as anoxia, results in antioxidant depletion or slowered turnover and hence increased oxidative damage on re-oxygenation (Blokhina *et al*. 1999).

As a result, after 3 days of anoxia the activity was 65% higher than in the control roots. In the more anoxia tolerant rice, anoxia did not affect SOD activity (Chirkova *et al*. 1999). Similar results were reported by Pavelic *et al*. (2000) for potato cell cultures during a post-anoxic period: only 60% of the initial specific SOD activity remained after 3h of reoxygenation. In cereals the activity of SOD has been found to decline depending on the duration of the anoxic treatment, while in *Iris pseudacorus* a 14-fold increase was observed during a reoxygenation period (Monk *et al*. 1989). An increase in total SOD activity was also detected in wheat roots under anoxia but not under hypoxia. The degree of increase positively correlated with duration of anoxia (Biemelt *et al*. 2000). Induction of SOD activity under hypoxia by 40-60% in roots and leaves under hypoxia of *H. vulgare* was shown by Kalashnikov *et al*. (1994).

Hence, investigations of SOD activity in different plant species under hypoxia (submergence) and/or anoxia have resulted in contradictory observations. The explanation can be found in different tolerance to anoxia between species and experimental setup (e.g. a prolonged reoxygenation period in the case of *Iris* spp., while in cereal roots activity of

the enzyme was determined immediately after anoxia). The formation of ROS already under hypoxic conditions and during reoxygenation could cause a rapid substrate overload of constitutive SOD, while induction could be probably by other factors (e.g. time, activity of downstream enzymes in the ROS-detoxification cascade, inhibition by the end product (H_2O_2) and consequences of anoxic metabolism). Observations on SOD activity in different plant species under several stress conditions (drought, salinity and high/low temperature) suggest that different mechanisms may be involved in oxidative stress injury (Yu and Rengel 1999a, 1999b). Activation of oxygen may proceed through different mechanisms, not necessarily producing a substrate for SOD. It is well known that flooding stress causes a decrease in water transport from the roots to leaves resulting in stomatal closure and water stress in the leaves. In that case light stress can lead to the formation of highly reactive singlet oxygen ($^1O_2$). Changes in O_2 electronic configuration can lead to the formation of highly reactive singlet oxygen ($^1O_2$). Comparison of water stress effects in tolerant and intolerant wheat genotypes suggests that different mechanisms can participate in ROS detoxification. For example, water stress leads to increased SOD activity in wheat but it was deduced that not SOD but ascorbate oxidase and catalase were the limiting factors in drought tolerance of susceptible wheat genotypes (Sairam *et al*. 1998). In another experiment, oxidative stress conditions combined with cold acclimation of cold-resistant and non-resistant wheat cultivars, SOD activity in the leaves and in the roots was unaffected by the low temperature treatment but plants exhibited higher guaiacol peroxidase activity (Scebba *et al*. 1998). Inefficiency of ROS detoxifying enzymes (SOD, CAT, ascorbate peroxidase and non-specific peroxidase) has been shown under water deficit-induced oxidative stress in rice (Boo and Jung 1999). In this paper a decrease in enzymatic activity was accompanied by lipid peroxidation (LP), chlorophyll bleaching, loss of AA reduced glutathione (GSH), α-tocopherol and carotenoids in stressed plants. The authors suggested the formation of a certain strong prooxidant, which is neither superoxide nor H_2O_2 under the conditions of water deficit (Boo and Jung 1999). The ability of plants to overcome oxidative stress only partly relies on the induction of SOD activity and other factors can regulate the availability of the substrate for SOD: diversification of the pathways of ROS formation, compartmentalization of oxidative processes (charged ROS cannot penetrate the membrane) and compartmentalization of SOD isozymes. It is also possible that in different plant species and tissues different mechanisms are involved in the protection against oxidative stress.

Catalases and peroxidases. Catalases and peroxidases are important enzymes present in the intercellular spaces, where they can regulate the level of H_2O_2 (reviewed by Willekens *et al*. 1995). Catalase functions through an intermediate catalase-H_2O_2 complex (called Compound I) and produces water and dioxygen (catalase action) or can decay to the inactive Compound II. In the presence of an appropriate substrate Compound I drives the peroxidatic reaction. Compound I is a much more effective oxidant than H_2O_2 itself, thus the reaction of Compound I with another H_2O_2 molecule (catalase action) represents a one-electron transfer, which splits peroxide and produces another strong oxidant, the hydroxyl radical OH• (Elstner 1987). OH• is a very strong oxidant and can initiate radical chain reactions with organic molecules, particularly with PUFA in membrane lipids.

Under anoxia a differential response of the peroxidase system has been observed in coleoptiles and roots of rice seedlings. A decrease in activities of cell wall-bound guaiacol and syringaldazine peroxidase activities was reported, while soluble peroxidase activity was not affected in coleoptiles. In contrast anoxia-grown roots showed an increase in the cell wall-bound peroxidases (Lee and Lin 1995). Acclimation to anoxia has been shown to be dependent, at least partly, on peroxidases, which are up-regulated by anoxic stress in soybean cell cultures (Amor *et al*. 2000). In rice seedlings ADH and SOD activities responded non-significantly to submergence, while catalase activity increased upon re-oxygenation (Ushimaru *et al*. 1999).

Phospholipid hydroperoxide glutathione peroxidase. A key enzyme in the protection of membranes exposed to oxidative stress is the phospholipid hydroperoxide glutathione peroxidase (PHGPX). It is inducible under various stress conditions. PHGPX can also react with H_2O_2 but this is a very slow process. The enzyme catalyses the regeneration of phospholipid hydroperoxides at the expense of GSH and is localised in the cytosol and the inner membrane of mitochondria of animal cells. A cDNA clone homologous to PHGPX has been isolated from tobacco, maize, soybean, and *Arabidopsis* (Sugimoto *et al*. 1997). The PHGPX protein and its encoded gene *csa* have been isolated and characterised in citrus. It has been shown that *csa* is directly induced by the substrate of PHGPX under heat, cold and salt stresses, and that this induction occurs mainly via the production of ROS (Avsian-Kretchmer *et al*. 1999). As ROS production increases also after flooding or anoxia, it is probable that the expression of this gene is induced after flooding stress.

Roles of ROS in signaling during hypoxic or anoxic stress

ROS are formed constitutively as by-products of oxidative metabolism. In most cases imposition of stress results in a shift in the redox balance towards oxidation. However, under hypoxic or anoxic stress, the redox balance is first shifted to reducing conditions and only after reaeration oxidations re-emerge and reactive oxygen species are formed. This is especially true in anoxia sensitive plants as their antioxidative capacity decreases during low oxygen conditions (Blokhina *et al*. 2000), and hence ROS formation may be enhanced after reoxygenation (Blokhina *et al.* 2001). These changes are brought about by enhanced ROS formation and/or by a decline in antioxidant capacity. A disturbed redox balance can itself be an inducing signal for defence mechanisms. Under normal physiological conditions (PCD during aerenchyma formation, stomatal movements) plant cells are able of controlled production of ROS as signaling molecules. Implication of ROS and particularly H_2O_2 in signaling has been shown in a number of abiotic stress responses such as oxygen deprivation, cell cycle regulation, cell death and wounding response (as reviewed in Blokhina *et al*. 2003 and Pitzschke *et al*. 2006) and can be transduced e.g. through protein cysteine oxidation (Cross and Templeton 2006).

Monitoring the expression of over 14,000 genes in catalase-deficient tobacco (CAT1AS) under H_2O_2-inducing exposure to high light has revealed transcriptional responses that mimic those of both biotic and abiotic stresses such as low oxygen stress. Clustering and sequence analysis has revealed induction of genes responsible for hormonal biosynthesis, pathogen defense, mitochondrial metabolism, vesicular trafficking, proteolysis and cell death (Vandenabeele *et al*. 2003). The latter events are meaningful also in the context of PCD in aerenchyma formation. The role of H_2O_2 (and NO) has been studied further in the CATIAS mutant by Zago and coworkers (2006) and their work clearly points to PCD regulation.

It is still not fully understood how H_2O_2 signals are perceived and transduced in aerenchyma formation. In maize roots the appearance of superoxide anions and hydrogen peroxide has been shown in cortical cells, which degenerate to form aerenchyma through programmed cell death (Bouranis *et al.* 2003). In pea (*Pisum sativum*) roots the imposition of flooding has been shown to lead to programmed cell death as demonstrated with the TUNEL method and by DNA laddering in procambial and ground meristem tissues (Gladish *et al.* 2006). Although we do not know yet how H_2O_2 acts in aerenchyma formation or in the induction of

protective events under flooding stress, it has known functions in related events. It has been shown that H_2O_2 is a potent inducer of specific mitogen-activated protein kinase kinase kinase (ANP1) in *Arabidopsis.* ANP1 initiates a phosphorylation cascade by mitogen-activated protein kinases (MAPK), which in turn lead to the induction of oxidative stress responsive genes (Kovtun *et al.* 2000). In another study, H_2O_2 exposure of *Arabidopsis* cells led to changed expression levels of 175 genes, of which 113 coded for proteins with antioxidant functions or were related to stress responses (Desikan *et al.* 2001).

The first redox-sensitive transcription factor that has been described in plants is NPR1, which acts as a regulator of plant systemic acquired resistance (SAR) (Mou *et al.* 2003). NPR1 function depends on ROS-mediated oxidation of reduced cysteine residues in a similar manner to *E. coli* OxyR and yeast Yap1 (as reviewed in Pitzschke *et al.* 2006). Another transcription factor that has been characterized recently is TaMYB1 from wheat roots and it is expressed during hypoxic conditions (Lee *et al.* 2007b). MYB transcription factors are known to be involved in abiotic stresses and the Myb binding site is vital for the anaerobic expression of the GapC4 promoter in tobacco (Geffers *et al.* 2001) and for the induction of ADH1 in *Arabidopsis* (Hoeren *et al.* 1998). Another transcription factor, NRF2, has also been shown to act in oxidative stress in mammalian and yeast cells, but it remains to be seen whether it is present in plant tissues (Karapetian *et al.* 2005).

H_2O_2 is known also to act as a signaling molecule in defense against pathogens (Desikan *et al.* 2001), in growth and morphogenesis through the cell cycle, and in responses to many plant hormones such as ethylene and abscisic acid, which have known functions in plants under flooded conditions (Overmyer *et al.* 2003). It has also been shown that H_2O_2-induced MAPK cascade in *Arabidopsis* represses auxin-inducible gene expression (Kovtun *et al.* 2000). However, it is known that the oxidative burst and cognate redox signaling work in a signal network that functions independently of ethylene, salicylic acid (SA) and methyl jasmonate (Me-JA) but is dependent on MAPKK activity (Grant *et al.* 2000).

DAVIES-ROBERTS pH-STAT THEORY

This concept, which was reviewed by Fox *et al*. (1995a, 1995b) and Ratcliffe (1997), was initially suggested by Davies *et al*. (1974), who studied the time-course of lactate and ethanol accumulation in cell-free extracts from pea seeds. According to this concept, the acidification of the cytoplasm during the first phase of anaerobiosis due to lactic fermentation results in inhibition of lactate dehydrogenase which exhibits optimum functioning at neutral pH and simultaneous activation of pyruvate decarboxylase (which has optimum activity at low pH). As a result, switching from lactic to ethanolic fermentation occurs. In organisms that cannot switch to ethanolic fermentation, further lactate accumulation leads to lowered cytoplasmic pH and eventually cell death due to cytoplasmic acidosis. Later studies, using NMR, confirmed the pH-stat theory (Roberts *et al.* 1982, 1984a, 1984b, 1985; Fan *et al.* 1988; Menegus *et al.* 1991; Xia and Roberts 1994; Fox *et al.* 1995a; Fan *et al.* 1997; Ratcliffe 1997; Chang *et al.* 2000; Fan *et al.* 2003). In particular, Roberts *et al.* (1982) used the pH-stat theory to explain results obtained with detached maize roots subjected to anaerobic stress in that there was a strong correlation between increased levels of lactate ions, cytoplasmic acidification and shortly followed by root tip death. When using weakly permeating bases and acids, researchers can change the cytoplasmic pH and induce a switch from one type of fermentation to another (Fox *et al.* 1995a), thus also arguing for the pH-stat theory. Thus, according to Davies-Roberts' concept (Davies 1980; Roberts *et al.* 1982, 1985), the acidification of the cytoplasm induces damage and eventually death of intolerant plants, whereas anoxia-tolerant plants control this acidification process by switching from lactic- to ethanolic fermentation.

However, the results of other researchers sometimes contradicted those of Davies and Roberts. In particular, it was shown that switching from lactic to ethanolic fermentation during the early period of anaerobiosis did not occur in all plant species. For example, in barley roots, lactic fermentation under anaerobic conditions lasted for four days (Hoffman *et al.* 1986). When studying the induction of alcoholic and lactic fermentation in various organs of different plants (pea and rice roots, pea embryos, apple fruit, and *Acer platanoides* leaves) after their transfer from aerobic to anaerobic environment, Andreev and Vartapetian (1992) concluded that there is no single universal mechanism for the induction of alcoholic and lactic fermentation. Kennedy *et al.* (1992) suggested that the Davies-Roberts theory could not be applied to plants with true tolerance to anoxia, such as rice and *Echinochloa*. Finally, in studies performed with maize roots (Saint-Ges *et al.* 1991) cytoplasm acidification coincided in time with the hydrolysis of nucleotide triphosphates but not with lactate accumulation.

Along with changes in the cytoplasmic pH, the application of NMR permitted the monitoring of biochemical conversions of organic compounds *in vivo* under conditions of hypoxia and anoxia. In particular, following the ^{13}C-acetate conversion under anoxic conditions showed label incorporation into citrate, glutamate, γ-aminobutyric acid, and succinate. Moreover, it was shown that the tricarboxylic acids and glyoxylate cycles function partially under anoxia (Fan *et al.* 2003).

Thus, changes and regulation of cytoplasmic pH occur not only as a result of lactate synthesis and nucleotide triphosphate hydrolysis but also because of the functioning of other anaerobic biochemical processes, including those catalyzed by glutamate dehydrogenase and malate decarboxylase. As a result, protons are consumed and pH of the cytoplasm is controlled. The enzymes responsible for the synthesis of alanine (Good and Crosby 1989) and γ-aminobutyric acid (Ford *et al*. 1996) are also actively involved in this process.

Some authors also consider the role of nitrate as a terminal acceptor of electrons during NAD^+ recyclization (Fan *et al.* 1988). This protective role of nitrate was also demonstrated under anoxia in electron-microscopic studies (Vartapetian and Polyakova 1999; Polyakova and Vartapetian 2003). Fan *et al.* (1988) believe that the process of cytoplasm acidification is suppressed by nitrate accepting protons. In some plants, lactate removal from the cells also favors reduced cytoplasm acidity (Rivoal and Hanson 1994).

Thus, the application of NMR technology considerably facilitated the *in vivo* observation not only of cytoplasmic pH changes but also of the processes of cell carbon and nitrogen cell component interconversions under conditions of anaerobic stress (Ratcliffe 1997; Fan *et al.* 2003).

Finally, in several studies, it was shown that when plants are transferred from aerobic to anaerobic environments, lactic and ethanolic fermentation do not occur successively, as is predicted by the pH-stat theory of Davies-Roberts, but rather simultaneously (Andreev and Vartapetian 1992). Alternatively anaerobic respiration functions essentially without lactic fermentation (Menegus *et al.* 1991).

Nevertheless, both alternative points of view, i.e., damage and death of plant cells under anaerobic stress as a result of cytoplasm acidification or due to energy shortage determined by substrate starvation or insufficient activity of glycolysis and fermentation are under active investigation and discussion (Chang *et al*. 2000; Summers *et al.* 2000; Gout *et al*. 2001; Sato *et al* 2002; Fan *et al*. 2003; Jackson and Ram 2003; Ismond *et al*. 2003; Loretti *et al*. 2003; Vartapetian *et al*. 2003; Felle 2005; Harada *et al*. 2005; Huang *et al*. 2005; Dixon *et al*. 2006; Felle 2006; Sachs and Vartapetian 2007).

Data in favor of the significance of energy metabolism for both cytoplasm acidification and plant tolerance was obtained by Xia *et al*. (1995). The authors showed that mannose and NaF partially suppressed the rate of anaerobic fer-

mentation (measured by ethanol accumulation), which was primarily induced by hypoxia. This resulted in a decrease in the content of ATP and total adenylates below the levels found in roots that were not subjected to hypoxia or treated with an inhibitor. Nevertheless, these conditions did not reduce the tolerance to anoxia of acclimated roots as well as their capability to regulate cytoplasmic pH. The authors suggested that hypoxic pretreatment could somehow improve the affinity of key enzymes for ATP and help maintain cytoplasmic pH maintenance. One such possibility is an enhanced lactate release into the surrounding medium, which might help to avoid cytoplasm acidification (Xia and Saglio 1992). Xia *et al*. (1995) indicated that under anoxia, survival of roots subjected to acclimation and control of cytoplasmic pH does not essentially depend on the actual ATP level in the cell, whereas the rate of ATP synthesis has greater significance. Although in these experiments, the level of ATP and energy charge in the root cells subjected to acclimation by hypoxia and treatments with inhibitors decreased, the rate of fermentation, i.e., ATP generation under anoxia, was 2.5- to 4-fold higher than in non-acclimated control roots. The threshold level of the fermentation (ATP production) in experimental roots, below which the roots lost their resistance to anoxia, was 2.5-fold higher than in control, non-acclimated roots. The authors concluded that a critical level of glycolytic flow under anoxia evidently reflects a lower rate of ATP production required for the maintenance of cell viability. Experiments by Generosova *et al.* (1998) with detached shoots of rice seedlings showed that exogenous cytoplasm acidification by exogenous application of a weak acid in fact markedly inhibited anaerobic growth of such flood-tolerant organs such as rice coleoptile. This "acid" effect could be weakened substantially by stimulating cell energy metabolism under anaerobic conditions with exogenous glucose. These results are in a good agreement with observations made on maize root tips and *Acer pseudoplatanus* cell cultures by NMR (Saint-Ges *et al.* 1991; Gout *et al.* 2001). It was shown that when plant cells were transferred from aerobic to anaerobic conditions, a simultaneous decrease in the cytoplasmic pH and the nucleotide triphosphate pool occurs. The authors believed that a sharp drop in pH during the early stages of anaerobiosis occurs because of nucleotide triphosphate hydrolysis. When anoxic A. *pseudoplatanus* cells were fed by glucose, the cytoplasmic pH partially increased due to ATP synthesis in the process of enhanced ethanolic fermentation.

ALTERNATIVE ELECTRON ACCEPTORS

Nitrate reduction into nitrite and ammonia under anoxia is considered by some researchers as a compensatory mechanism of NADH oxidation (Reggiani *et al.* 1985a; Fan *et al.* 1988; Ivanov and Andreev 1992; Fan *et al.* 1997; Antonacci *et al*. 2007). Such oxidation helps to escape cytoplasm acidification because nitrate reduction is proton consuming process functioning as biochemical pH-stat (Roberts *et al.* 1985; Fan *et al.* 1997; Oberson *et al.* 1999; Libourel *et al*. 2006). It is also believed that glycolysis and fermentation, i.e., anaerobic cell energy metabolism, could be accelerated by such way (Reggiani *et al.* 1985a, 1985b). According to other researchers, a positive physiological role of nitrate under hypoxia is no evident (Saglio *et al.* 1988). Finally, based on investigations of exogenous nitrate action on growth and energy metabolism in rice, pea, and wheat seedlings, it was concluded that nitrate effects on plant adaptation to anaerobic stress are negative (Ivanov and Andreev 1992).

In the study of Fan *et al.* (1988), it was shown that exogenous nitrate reduced ethanol accumulation in maize roots under conditions of anoxia whereas in other studies (Reggiani *et al.* 1985a, 1985b; Mattana *et al.* 1993; Müller *et al.* 1994), nitrate stimulated anaerobic respiration in the rice and *Carex* roots. Botler and Kaiser (1997) did not observe any enhancement of ethanolic fermentation in barley roots under anaerobiosis, although the activity of nitrate reductase increased substantially. Electron-microscopic examinations of exogenous nitrate effect on the ultrastructure of rice coleoptile and wheat root mitochondria under conditions of strict anoxia (Vartapetian and Polyakova 1999; Polyakova and Vartapetian 2003) allowed the conclusion that nitrate exerts a protective action under these extreme conditions. Thus, when detached roots and coleoptiles were incubated under anaerobiosis in the absence of nitrate, mitochondria were destroyed in 6-9 h and 24-48 h, correspondingly, whereas, in the presence of nitrate and under the same experimental conditions, mitochondria remained intact even after 9 h (root) and 48 h (coleoptile) of anaerobic incubation (Polyakova and Vartapetian 2003). The protective effects of nitrate in rice coleoptiles were evidently related to the stimulation of energy metabolism because, in rice coleoptiles under anoxia, ethanolic fermentation prevails but not lactic fermentation leading to proton accumulation (Menegus *et al.* 1991). On the other hand, in rice coleoptile exposed to anoxia, nitrates are reduced to NH_4 and amino acids (Mattana *et al.* 1993), whereas, in roots nitrates are reduced to nitrite (Botrel and Kaiser 1997).

The beneficial effect of nitrate in relation both cytoplasmic acidification and plant survival under anoxia was recently confirmed (Stoimenova *et al*. 2003; Allegre *et al*. 2004; Libourel *et al*. 2006; Antonacci *et al*. 2007). However, there is some doubt in relation to above mentioned explanations of mechanisms responsible for protective effect of nitrate in the absence of molecular oxygen. Moreover, basing on accumulated experimental data Libourel *et al.* (2006) concluded that the reason for the beneficial effect of nitrate on pH regulation under anoxia is unknown. The results of *in vivo* ^{31}P NMR spectroscopy investigation of both nitrate and nitrite effects on cytoplasma acidification of *Zea mays* root segment under anoxia demonstrated unexpectedly that beneficial effect of nitrate should be explained by anaerobic reduction of nitrite to nitric oxide but not nitrate to nitrite (Libourel *et al.* 2006).

The physiological role of class 1 haemoglobin in oxidation of NO, generated in plant cell under hypoxia as a result of nitrate reduction (haemoglobin-based nitrate recycling), we have discussed in the previous section of this review.

Kennedy *et al.* (1991) believed that, along with nitrate, anaerobically synthesized lipids could serve as alternative terminal acceptors of electrons and protons under conditions of anaerobic stress. In addition, it was suggested that unsaturated fatty acids (FAs) could serve as proton acceptors under anoxia (Zs.-Nagy and Galli 1977; Chirkova 1988). Henzi and Brändle (1993) showed that, during a 70-day-long anaerobiosis exposure of the rhizomes of some plants inhabiting flooding soils, the degree of FA saturation increased and the amount of unsaturated FAs, especially linolenic acid, was reduced. The role of anaerobically synthesized lipids, as alternative electron acceptors for plants under anaerobic stress was studied in experiments on the weed growing in rice fields *Echinochloa phyllopogon* (Kennedy *et al.* 1991; Fox *et al.* 1994) for which seeds, as for rice seeds, germinate easily under anoxic conditions (Kennedy *et al.* 1980). The authors showed that during anaerobic germination of *E. phyllopogon* seeds, primary leaves actively accumulated lipid bodies (spherosomes). It was concluded that lipids synthesized *de novo* under anoxia served as acceptors of electrons and protons. The authors considered this phenomenon as a biochemical mechanism of adaptation to anoxia of such tolerant plants such as *E. phyllopogon* and rice seedlings (Kennedy *et al.* 1991; Fox *et al.* 1994). In fact, Vartapetian *et al.* (1978c) and Kennedy *et al.* (1991) demonstrated experimentally that lipid precursors, ^{14}C-acetate and ^{3}H-glycerol, were incorporated under anoxia into the molecules of phospholipids, glycolipids, and neutral lipids of primary leaves of rice and *E. phyllopogon* seedlings. However during the course of anaerobic lipid biosynthesis of lipids in rice coleoptiles the lipid precursor ^{14}C-acetate was only incorporated only in saturated but not in unsaturated fatty acids (Vartapetian *et al.* 1978c). In experiments with three- and seven-day old rice coleoptiles

Brown and Beevers (1987) also demonstrated that no significant increase occurred in unsaturated fatty acids took place during anaerobic growth of coleoptiles. Only a small increase in saturated fatty acids could be detected under anoxia. Subsequent electron-microscopic and biochemical studies with anaerobically germinated rice seeds (Vartapetian *et al.* 2003; Generozova and Vartapetian 2005) showed that under conditions of anaerobiosis, rice coleoptiles did not accumulate lipid bodies and that the level of FAs did not markedly increase. FA saturation was also not observed: index of their saturation was practically similar before and after long-term anaerobic incubation of germinating seeds. It was concluded that neither lipid unsaturated FAs of lipids nor anaerobically synthesized lipids function as terminal acceptors of electrons as an alternative to molecular oxygen in rice seedlings under anaerobic conditions. Studies of various lipid classes in rice seedlings grown under aerobic and anaerobic conditions (Vartapetian *et al.* 1978b) also favor this point of view, to some degree. In these latter experiments, no substantial differences in qualitative and quantitative composition of lipid FAs between seedlings grown under contrasting conditions was not found. Hence, the results of the aforementioned experiments with incorporation of ^{14}C-acetate and ^{3}H-glycerol into various lipid classes under anoxia (Vartapetian *et al.* 1978c; Kennedy *et al.* 1991) can be considered as a demonstration of saturated FA turnover in lipids without a corresponding lipid accumulation or FA saturation.

Thus, in contrast to conclusions of Kennedy and coworkers (Kennedy *et al.* 1991; Fox *et al.* 1994) and some other researchers (Zs.-Nagy and Galli 1977; Chirkova 1988), it was concluded that under conditions of anaerobic stress, neither lipid synthesis and accumulation nor FA saturation in rice seedlings could be considered as an alternative mechanism of electron acceptation and plant adaptation to anaerobic stress (Vartapetian *et al.* 2003; Generozova and Vartapetian 2005).

DEMONSTRATION OF ADAPTATION SYNDROME IN PLANTS UNDER ANAEROBIC STRESS

The exposure of plant organs and tissues that are sensitive to anaerobic stress at oxygen deficiency results in characteristic changes primary in the mitochondrial membrane ultrastructure; namely, the cristae disappear and mitochondria themselves are subjected to swelling (Vartapetian *et al.* 2003). Early changes in the mitochondrial structure are reversible: after a plant is transferred back to aerobic conditions, their ultrastructure and capacity for oxidative phosphorylation is completely restored (Andreev *et al.* 1996). During longer anaerobiosis treatments, destructive changes in mitochondria become more pronounced, mitochondrial degradation becomes irreversible and the cells die. When plant cells or organs are tolerant to anoxia i.e., rice coleoptiles (Vartapetian *et al.* 1978a, 2003), or the resistance to anoxia of not tolerant plant organs is increased artificially by feeding with exogenous sugar (Vartapetian *et al.* 1977), there is no destructive changes in mitochondria under rather long-term oxygen deficiency. However, mitochondria will often acquire an elongated shape, and/or the cristae are positioned in parallel rows (Vartapetian *et al.* 1977, 2003).

A more detailed electron-microscopic examination of the initial stage of anaerobiosis revealed unexpected rearrangements of the mitochondrial ultrastructure, which were not noticed in earlier experiments. In maize and wheat seedlings as soon as 15-30 min and in pea roots even within 2 min after their transfer from aerobic to anaerobic environment, an obvious destruction of some mitochondria was observed (Generozova *et al.* 1984; Vartapetian *et al.* 1987; Andreev *et al.* 1991). After 60-90 min, almost all mitochondria swelled and lost their cristae. However, during an extended exposure to anaerobiosis, mitochondria did not continue to degrade but, in contrast, completely restored their initial ultrastructure. By 3-5 h of anaerobiosis, the mitochondrial matrix became denser and cristae reappeared. This state of ultrastructure was maintained for several hours. Following this period, a new wave of mitochondrial destruction occurred, which after 24 h for leaves and within 12-24 h for roots, resulted in irreversible degradation of mitochondria and other cell organelles.

In order to elucidate possible molecular mechanisms of such unexpected ultrastructural rearrangements of mitochondrial membranes, we performed an anaerobic incubation of wheat leaves in the presence of exogenous glucose because, in earlier experiments, as was aforementioned, such feeding enhanced glycolysis and fermentation, thus maintaining a high level of the cell energy status and intact ultrastructure of mitochondria. In fact, when feeding with glucose, there were no signs of mitochondrial destruction after either 30, or 60, or 90 min of anaerobiosis (Vartapetian *et al.* 2003). These results of these experiments seemingly indicate that early mitochondrial membrane destruction in the absence of exogenous glucose occurs due to substrate starvation. However, subsequent restoration of mitochondrial ultrastructure under lasting anaerobic incubation in the absence of exogenous glucose contradicts this supposition about substrate starvation.

A possible hypothesis to explain this phenomenon is that, with increasing duration of anaerobic incubation, anaerobic proteins, including enzymes of glycolysis and fermentation, are synthesized, which accelerates glycolysis and ATP generation and, correspondingly, favors restoration of the mitochondrial ultrastructure. To verify this hypothesis, plant anaerobic incubation was performed in the presence of 10^{-5} M cycloheximide, thus inhibiting the synthesis of *de novo* proteins. Under these conditions, mitochondria swelled in 30-90 min. However, as distinct from treatment in the absence of cycloheximide, subsequent restoration of their ultrastructure in 3-5 h of anaerobic incubation was not observed. In contrast, within 6-9 h, irreversible degradation of mitochondria occurred (Vartapetian *et al.* 2003). The results of these experiments lead to a model showing that cell energy metabolism plays a key role in early destruction and subsequent regeneration of mitochondrial ultrastructure. During 3-5 h, both feeding with glucose and the synthesis of anaerobic proteins (most of them are enzymes of glycolysis and fermentation and also other related processes of carbohydrate metabolism) favor corresponding enzyme-substrate interaction. At the same time, ATP generation is enhanced, which favors the restoration of mitochondrial membrane fine structure. It should be noted that Van Toai and Bolles (1991) observed a similar situation when studying post-anaerobic *Glycine max* cell injury with reactive oxygen species. The authors transferred the cells after 1-2 h of anaerobiosis to an aerobic environment and observed that they were damaged by reactive oxygen species. When the cells were transferred to an aerobic environment after 3-5 h of anaerobiosis, damage was insignificant or absent, evidently due to anaerobic synthesis of SOD, a scavenger of oxygen radicals.

The above-described phenomenon of ultrastructural rearrangements of mitochondrial membranes, which was observed initially in experiments with various maize organs (Generozova *et al.* 1984), was also described for the roots of anaerobically incubated maize seedlings (Aldrich *et al.* 1985). However, reversible destruction of mitochondrial membranes occurred in these experiments of Aldrich *et al.* (1985) during 8-26 h of anaerobiosis, whereas, in experiments of Generozova *et al.* (1984) similar rearrangements occurred during much shorter exposures to anaerobic conditions while under such long-term anaerobiosis, mitochondria and other cell organelles in the maize roots displayed obvious signs of degradation.

The phenomenon of reversibility of mitochondrial membrane destruction and restoration under extreme conditions of continuous anaerobic stress is of some historical interest as well. The results of these experiments demonstrated at the subcellular level the applicability to plants of the concept of “general adaptation syndrome”, which was put forward by the physician Hans Selye about 60 years ago

for animals and human (Selye 1950), to understand the putative mechanisms functioning under stress conditions. According to Selye, human and animal responses to unfavorable conditions consists of three successive stages: the state of unspecific stress or "alarm" stage (in our case, reversible destruction of mitochondrial membranes in leaves and roots); "adaptation" state (in our case, the recovery of initial mitochondrial ultrastructure in leaves and roots); and finally "exhaustion" state (in our case, irreversible degradation of mitochondrial membranes in leaves and roots at more prolonged anaerobic incubation).

GENETIC AND CELLULAR ENGINEERING

Taking into account the role of glycolysis and alcoholic fermentation in plant adaptation to hypoxia and anoxia, attempts were made to increase the rate of ethanolic fermentation and thus plant tolerance by genetic engineering manipulations (Bücher *et al.* 1994; Tadege *et al.* 1998; Quimio *et al.* 2000; Rahman *et al.* 2001; Ismond *et al.* 2003). Thus, in experiments of Bücher *et al.* (1994), transgenic tobacco plants were obtained by insertion of the pyruvate decarboxylase (PDC) gene from the obligatory anaerobic bacterium *Zymomonas mobilis* into the plant genome. The transgenic exhibited an increased content of PDC protein in leaves and an increased activity of the enzyme *in vitro* and *in vivo*. Correspondingly, during the first 2-4 h of anoxia, the leaves of the transgenic tobacco accumulated more acetaldehyde (by 10-35 times) and ethanol (by 8-20 times) than the leaves of wild-type plants. However, the plants did not display an improved tolerance to anoxia. Tadege *et al.* (1998) also inserted the PDC gene from *Z. mobilis* into the tobacco genome. The accumulation pattern of the products of ethanolic fermentation in plant roots differed somewhat from that observed earlier by Bücher *et al.* (1994). Since the initial activity of pyruvate decarboxylase (PDC) in wild-type tobacco roots was much higher than in leaves, the introduction of the bacterial gene resulted in an insignificant enzyme activation and, correspondingly, a lower accumulation of acetaldehyde and ethanol in the roots as compared with the leaves. Nevertheless, the acceleration of ethanolic fermentation in transgenic roots, in fact reduced root tolerance to anoxia. The authors supposed that an enhanced carbohydrate consumption in the process of accelerated glycolysis and fermentation exhausted tissues in substrates for glycolysis. Thus, substrate starvation caused a more rapid death of transgenic plants under anoxia. In fact, feeding of transgenic plants with exogenous sugars improved their tolerance to anoxia (Tadege *et al.* 1998).

Quimio *et al.* (2000), but not Rahman *et al.* (2001), reported an improved tolerance to submergence of transgenic rice seedlings over-expressing a PDC gene. The results obtained by Ismond *at al.* (2003) are more supportive. They manipulated the level of enzymes of alcoholic fermentation, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), in transgenic *Arabidopsis* plants. In contrast to the results of Tadege *et al.* (1998), the *Arabidopsis* with a transgenic PDC gene not only displayed an accelerated ethanolic fermentation but also a higher tolerance to hypoxia as compared to wild-type plants. In contrast to the PDC transgenic plants, *Arabidopsis* with a transgenic ADH gene exhibited an increase in the ADH activity that did not result in improved tolerance. Furthermore, in the *adh1* mutant, accumulation of acetaldehyde dropped sharply and plant tolerance to low-oxygen stress was strongly reduced. A high sensitivity of *Arabidopsis* ADH null mutants to hypoxia, as in the previous experiments of Schwartz (1969) in maize, could be induced by acetaldehyde accumulation, which quantity rose sharply in the cells devoid of ADH and, this correspondingly, lessens the possibility for reducing acetaldehyde and thus no longer protecting the cells from its toxic effects. Along with the acetaldehyde accumulation it is impossible to exclude the probable accumulation of pyruvate which could result, with the involvement of lactate dehydrogenase (LDH), in the accumulation of toxic amounts of lactate or acidosis. Ismond *et al*. (2003) performed experiments on plant feeding with 3% sucrose and showed that a sufficient supply of substrate to the plant helped to improve their tolerance to oxygen deficiency. This conclusion is in a good agreement with the results of earlier experiments (Vartapetian *et al.* 1977, 1978a) and subsequent data of others researchers (Saglio *et al.* 1980; Brändle 1985; Johnson *et al.* 1989; Waters *et al.* 1991; Hole *et al.* 1992; Xia and Saglio 1992; Xia *et al*. 1995; Ricard *et al.* 1998; Tadege *et al.* 1998; Loreti *et al.* 2003).

On the basis of their studies, Ismond *et al.* (2003) concluded that PDC activity is tightly related to the rate of carbon flow along the pathway of ethanolic fermentation and determines a tolerance to low-oxygen stress, i.e., PDC immediately controls ethanolic fermentation. Thus, the results obtained by Ismond *et al.* (2003) substantially supported the idea of a key role for energy metabolism in the true plant tolerance to anaerobic stress (Vartapetian *et al.* 1977, 1978a, 2003).

Another approach applied to create plants more tolerant to anaerobic stress has been the selection of cultured sugarcane *Saccharum officinarum* and wheat *Triticum aestivum* cell lines (Stepanova *et al.* 2002; Vartapetian *et al.* 2003). In these experiments, calli derived from the meristem of sugarcane and wheat embryos and grown under aerobic conditions on a modified Murashige and Skoog (MS) nutrient medium (Kharinarain *et al.* 1996) were then subjected to a stepwise selection under anoxia of increasing duration.

Based on the notion of a key role of carbohydrate and energy metabolism in plant cell tolerance to anaerobiosis (Vartapetian *et al.* 1977, 1978a) exogenous sugar was excluded from the MS nutrient medium during anaerobic incubation. This circumstance most likely had a decisive consequence for successive selection of more tolerant cells because, under these conditions, cell tolerance to the absence of oxygen was entirely determined by mobilization of endogenous carbohydrate reserves and their subsequent utilization in the processes of energy metabolism (glycolysis, fermentation). The presence of exogenous sugars in MS medium could substantially affect all these processes making even impossible the selection of tolerant cells. The results of electron-microscopic examinations and also a cell capacity for post-anaerobic growth showed that the cells selected in such a way were much more tolerant to anoxia than control, initial cells. Plants regenerated from such tolerant cells turned out to be more tolerant to soil anaerobiosis than the parent plants, which were used for callus production (Stepanova *et al.* 2002; Vartapetian *et al.* 2003).

CONCLUDING REMARKS

Studies on plant anaerobic stress during past decades confirmed and substantially developed the concept of two general strategies of plant adaptation to hypoxia and anoxia. Based on the scientific advances that demonstrated the key role of energy and related processes of carbohydrate mobilization and utilization in plant metabolic adaptation to oxygen deficiency, the first attempts were made to create plants more tolerant to anaerobic stress using biotechnological approaches (such as gene and cell engineering) for stimulation and regulation of plant energy metabolism (glycolysis and fermentation).

This review paid special attention to the second general strategy of plant adaptation to oxygen deficiency in the environment by formation of aerenchyma and distant transport of molecular oxygen, i.e. by avoidance of anaerobiosis. Accordingly, special attention is paid to mechanism of aerenchyma formation, which considerably facilitates O_2 transport from aerated plant parts to the organs located in an anaerobic environment. Progress in the studies of aerenchyma formation and oxygen transport from aerated plant parts to the roots located in an anaerobic environment resulted in new essential evidence of the pivotal role of distant transport of molecular oxygen, rather than root metabolic adaptation, in the maintenance of vital functions of the

plants inhabiting submerged and waterlogged soils. Marked success was also achieved in identifying of signaling systems and molecular mechanisms that function both in tolerant and intolerant plants under hypoxia and anoxia in the process of aerenchyma formation.

Progress has been made in the studies of the role of both post-anaerobic oxidative stress in plants, and of protective low molecular weight systems and enzymatic mechanisms operating under such stress conditions. Both of these mechanisms play an important role under normal aerobic conditions and especially during post-anoxic recovery of plant tissues. In addition to the well-known antioxidants, plants contain numerous small molecular compounds, which have their main functions elsewhere in metabolism, but which have antioxidative properties. Such compounds, e.g. of phenolic origin, may have yet undiscovered significance in the protection of plant cells against oxidative damage.

The chemistry of the production of various ROS and RNS has been studied in detail in plants under normal conditions as well as under biotic and abiotic stresses such as low oxygen availability, but many molecular level interactions are still unclear. At the moment research efforts are concentrating on the signaling roles and routes of the various reactive species and their crosstalk, not only under different stress conditions but also in the regulation of developmental events. Some details of this obviously intricate signaling network are beginning to emerge, while others, such as the probable ROS or RNS interaction with many other transcription factors than just NPR1, remain elusive.

During the last decades considerable advances were also achieved in NMR-studies of the role of lactate and ethanolic fermentation in plant adaptation to anaerobic stress. A predominant role of ethanolic fermentation has become evident in both anaerobic energy generation and intracellular stabilization. Nevertheless, some experimental evidence has been accumulated suggesting that in addition to lactate and ethanolic fermentation, other biochemical processes associated with electron and proton acceptance have an important part in stabilization of cellular environment under hypoxic and anoxic stresses. Specifically, the role of nitrate as one of such terminal electron acceptors was rather convincingly demonstrated in some studies. Lastly, experiments on the plants exposed to anaerobic stress have for the first time visualized and demonstrated on the subcellular level the phenomenon of adaptation syndrome in plants and possible mechanisms of its realization on molecular level.

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